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Role of Rho GTPase in the Endothelin-1-Induced Nuclear Signaling

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Binding of Endothelin-1 (ET-1) to its heterotrimeric G protein-coupled receptors stimulates various signaling cascades involving the activation of phospholipase C-β, phospholipase D, protein kinase C (PKC), tyrosine kinases, Ca^{2+}/calmodulin-dependent kinase (CaMKs), and Ras, a small molecular weight G-protein, but, the role of Rho GTPase remains unclear. In this project, we examined whether RhoA contributes to the ET-1-induced signaling cascade to *c-fos* SRE activation in Rat-2 fibroblast cells. Our results demonstrate that Rho activation is critical for the signal transduction of ET-1 to *c-fos* SRE.

Endothelin-1 (ET-1) binds to heterotrimeric G protein-linked receptors and regulates a wide range of biological actions such as cell growth, differentiation, cytoskeletal reorganization, vasoconstriction, and gene expression in a variety of cell types (1-4). One of the earliest nuclear responses by ET-1 is transcriptional activation of immediate early genes including *c-fos* protooncogene (5-6). Based on the recent report by Herman and Simonson, Ras pathway contributes to the nuclear signaling of ET-1 to transcriptional activation of *c-fos* through the serum response element (SRE) cis-element in glomerular mesangial cells (7). In more recent paper, the role of voltage-insensitive Ca^{2+} channels and Ca^{2+}/calmodulin-dependent protein kinase II (CaMK-II) were suggested in the ET-1 signaling pathway to *c-fos* activation (8). It was recently suggest that Ras-related proteins, Rho family GTPases, are involved in the signal transduction pathway of SRE activation via serum response factor (SRF) (9). In the present study, we examined whether RhoA activation contributes the signaling cascades of ET-1 to *c-fos* SRE in Rat-2 fibroblast cells. By transient transfection analysis of SRE-luciferase reporter gene, we demonstrate, for the first time, that RhoA activity is critical in the signaling cascade of ET-1 to *c-fos* SRE.

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MATERIAL AND METHODS

Chemicals and reagents. Phorbol 12-myristate 13-acetate (PMA), lysophosphatidic acid (LPA), and endothelin-1 were obtained from Sigma Chemical Co. (St Louis, MO, USA). Epidermal growth factor (EGF) was purchased from Boehringer Mannheim GmbH (Mannheim, FRG). Fetal bovine serum (FBS), gentamycin, Dulbecco's modified eagle's medium (DMEM) were from Gibco-BRL (Gaithersburg, MD, USA). All other chemicals were from standard sources and were molecular biology grade or higher.

Plasmids and DNA manipulations. The reporter gene pSRE-Luc was derived from pFos-lcf (10) and it contains sequences −53 to +45 of the *c-fos* promoter upstream of the luciferase coding sequences with *c-fos* SRE oligonucleotide inserted at the −53 position. pEXV, pEXV-RacN17 were gifts from Dr. A. Hall. pEXV-RhoN19 plasmid was constructed by subcloning RhoAN19 fragment into EcoRI site of pEXV vector. RasN17 was gifts from Dr. Kumar (Schering-Plough Research Institute, NJ). All Rac, Rho, and Ras proteins were expressed as N-terminally 9E10 epitope-tagged derivatives under SV40 promoter.

Cell culture, transfections, and luciferase assay. Rat-2 fibroblast cells were obtained from the American Type Culture Collection (ATCC, CRL 1764). Cells were grown in Dulbecco's modified eagle's medium (DMEM) supplemented with 2 mM glutamine, 10% (Vol/Vol) fetal bovine serum (FBS) and gentamycin as described before (11). Transient transfection analysis was performed by calcium phosphate : DNA precipitation method (12-13). To control for variations in both cell numbers and transfection efficiency, all clones were cotransfected with pCMV-βGAL, an eucaryotic expression vector in which E. coli β-galactosidase (lacZ) structural gene is under the transcriptional control of the CMV promoter. Lysates prepared from the harvested cells were assayed for both luciferase activity and β-galactosidase activity, which was used as an internal standard to normalize the luciferase activity directed by the test plasmid. Luciferase activity was determined as described previously (14). Transfection experiments were performed in triplicate with two independently isolated sets and the results were averaged.

RESULTS AND DISCUSSION

As a first approach to study the role of RhoA in ET-1 signaling cascade to nucleus in Rat-2 fibroblast cells,

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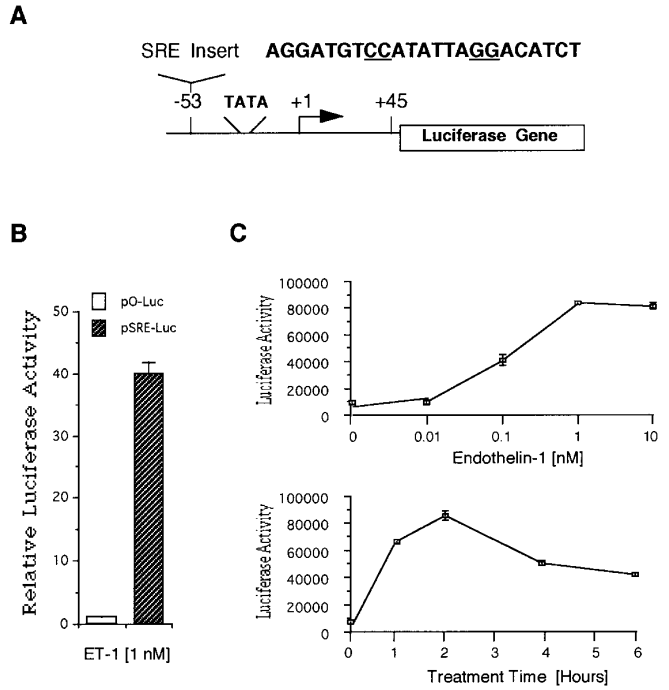

FIG. 1. ET-1 activates *c-fos* SRE in a dose and time-dependent manner. *A*, Diagram of pSRE-luciferase reporter gene plasmids that were used. The structure of construct containing SRE oligonucleotide sequences (23 mers) inserted to −53 position of the truncated *c-fos* promoter fused to luciferase gene is shown. *B*, Transient transfection assay with either pSRE-Luc or pO-Luc (vector without SRE insert). After transient transfections, Rat-2 cells were serum-starved in 0.5% FBS/DMEM for 36 hr before ET-1 treatment (1 nM). ET-1 was treated for 2 hr before cell harvest. The relative activation of pSRE-luciferases to pO-luciferase was calculated as described in materials and methods and histograms of the results of the luciferase are shown. Values were representative of multiple transfections. *C*, (top) Dose response of ET-1 on SRE activation. Various amounts of ET-1 (0, 0.01, 0.1, 1, 10 nM) were treated for 2 hr to serum-starved transfected cells. *C*, (bottom) Time-dependent response of ET-1 (1 nM) on SRE activation. Serum-starved Rat-2 cells were treated with ET-1 for the lengths of time indicated. Luciferase and β-galactosidase activities were measured as described in materials and methods.

we first determined whether ET-1 could activate *c-fos* serum response element (SRE). The SRE-stimulating activity by ET-1 was analyzed by transient transfection analysis of a reporter plasmid, pSRE-Luc containing *c-fos* SRE fused to luciferase coding sequences (see Fig. 1A). Following transient transfection, Rat-2 cells were serum-starved in DMEM containing 0.5% FBS for 36 hr before treating ET-1. SRE activation was monitored by luciferase activities normalized with co-transfected β-galactosidase activity. As shown in figure 1C, ET-1 stimulated the *c-fos* SRE-dependent reporter gene activity in a dose and time-dependent manner. A maximum luciferase activity occurred 2 hr after the addition of ET-1 and by 6 hr, the *c-fos* SRE luciferase level declined (Fig. 1C *bottom*). At 1 nM concentration of ET-1, a maximum increase in the luciferase activity was detected (Fig. 1C *top*). In a control experiment to demonstrate the specific action of ET-1 toward SRE, Rat-2 cells were transiently transfected with pO-Luc (vector without SRE insert). No stimulation of luciferase activity, however, was observed in response to ET-1 addition, suggesting that the ET-1 signal to SRE activation is specific (Fig. 1B).

ET-1 activates SRE in both TCF/Elk-1-independent and -dependent pathways. At the *c-fos* SRE, SRF forms a ternary complex with TCF (ternary complex factor), which cannot bind the SRE by itself (10). The Elk-1, one of TCFs, has been shown to regulate SRE in response to activation of the Ras-Raf-MEK-ERK (extracellular signal-regulated protein kinases) pathway (10). The TCF binding was not always required, however, especially for serum or lysophosphatidic acid (LPA)-induced SRE activation, indicating that there is TCF/Elk-1-independent signaling pathway as well as TCF/Elk-1-dependent pathway for the SRE activation (9). Similarly, Rho family GTPases have been shown to play a role in the signaling to SRE activation through TCF/Elk-1-independent pathway which probably involves the direct activation of SRF (9). In a previous study by Herman and Simonson, the Ras-linked pathway was reported to play a role in ET-1-induced SRE activation in mesengial cells, probably through TCF/Elk-1-dependent pathway (7). To determine whether ET-1-induced SRE activation is dependent on TCF/Elk-1-dependent signaling pathway in Rat-2 fibroblast cells, subconfluent Rat-2 cells were transiently transfected with a mutant SRE-luciferase plasmid defective in TCF/Elk-1 binding (pSREmt-Luc) and measured the luciferase activity after ET-1 treatment. The oligonucleotide in pSREmt-Luc plasmid contains two point mutations (*AGA* to *TGT*) which abolish TCF binding (Fig. 2A) (10). As shown in figure 2B, ET-1 activated SRE-mt slightly less than SRE-wild type (about 5.3 fold vs 3.8 fold), indicating that both TCF/Elk-1-dependent and -independent signaling pathways are probably involved in ET-1 signaling pathway to SRE in Rat-2 fibroblasts (Fig. 2B). In contrast, PMA (phorbol-12-myristate-13-acetate; 10 ng/ml) activated mutant SRE-dependent luciferase activity much less compared to wild type SRE (30% activation of SREwt-luciferase activity). LPA (lysophosphatidic acid; 10 μM), which was previously shown to activate SRE by TCF/Elk-1-independent pathway, stimulated both SRE mutant- and SRE wild type-dependent luciferase activity to the same level (7-8 fold) (9). Thus, from these results, it could be speculated that besides TCF/Elk-1-linked pathway, TCF/Elk-1-independent cascade exist to mediate ET-1 signal to SRE probably via Ras-independent manner.

RhoA is critical for the ET-1-induced signaling pathway to SRE activation. Previously, the potential role of RhoA in the signal transduction pathway of ET-1 in

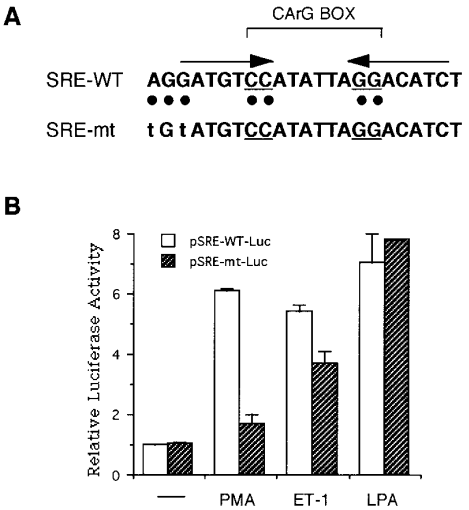

FIG. 2. ET-1-induced SRE activation is both dependent and independent to TCF/Elk-1. *A,* Sequences of wild type or mutant SRE oligonucleotide (23 mers) which were inserted to −53 position of the truncated *c-fos* promoter fused to luciferase gene. Also, the methylation interference pattern for SRF ternary complex with TCF is designated as closed circles. The mutant SRE has two point mutations (*AGG* to *TGT*) in the TCF-binding region that abolish the SRF/TCF ternary complex formation. *B,* Transient transfection assay with either pSREwt-Luc or pSREmt-Luc. PMA (10 ng/ml), ET-1 (1 nM), or LPA (10 μM) was treated for 2 hr before cell harvest. The relative activation of pSRE-WT-Luc to pSRE-mt-Luc was calculated and histograms of the results of the luciferase are shown.

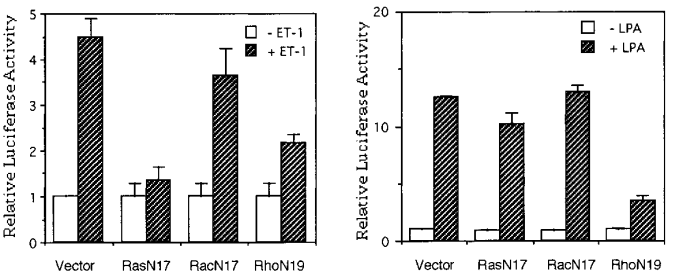

FIG. 3. Rho activity is critical for the ET-1-induced SRE activation. A reporter gene plasmid, pSRE-WT-Luc (3 μg) was transiently co-transfected with 5 μg of pEXV (vector), pRasN17, pRacN17, or pRhoAN19 plasmid. Total amounts of DNA were kept at 20 μg with calf thymus carrier DNA. Transfected cells were serum-deprived in DMEM containing 0.5% FBS for 36 hr before harvest. ET-1 (1 nM) or LPA (10 μM) was added 2 hr prior to cell harvest, and luciferase and β-galactosidase activities were measured. Values were representative of multiple transfections.

the cell has been suggested especially in the regulation of stress fiber and focal adhesion formations (15). For example, ET-1-stimulated focal adhesion kinase (FAK) tyrosine phosphorylation causes the rapid assembly of focal adhesions and stress fibers in Swiss 3T3 cells (15-16). Since actin stress fiber formation is known to be mediated by RhoA, the signaling connection linking endothelin-1 and RhoA activation could be expected (17-18). To determine whether RhoA activation contributes to the ET-1-induced SRE activation in Rat-2 cells, we used an expression vector encoding a dominant negative RhoA mutant (RhoAN19). As shown in figure 3A, co-transfection of either RasN17 (a dominant negative Ras) or RhoAN19 led to significant inhibition of SRE activation by ET-1. On the other hand, RacN17 (a dominant negative Rac) co-transfection showed little inhibition (Fig. 3A). In a control experiment, LPA (lysophosphatidic acid), which was previously shown to activate SRE via Rho mostly, was used and, as expected, LPA-induced SRE activation was inhibited only by RhoAN19, not by either RasN17 or RacN17 (Fig. 3B). Together, these results suggest an critical role of RhoA in the signaling pathway of ET-1 to SRE. Therefore, besides Ras/Raf-1/ERK cascade, RhoA-linked pathway is also important in the ET-1 signaling to *c-fos* SRE.

To further assess the role of RhoA in ET-1 signaling cascade to *c-fos* SRE, botulinum C3 transferase expression was used. Expression of C3 transferase has been shown to specifically inhibit Rho via ADP-ribosylation at Asn-41 (9). Cells were transfected with pEFplink (vector) or pEFC3 (C3 transferase), together with SRE-luciferase reporter gene to see whether C3 transferase expression blocks ET-1-induced SRE activation (9). We observed that C3 transferase blocked specifically ET-1-induced SRE activation without affecting EGF (50 ng/ml)-induced SRE activation significantly (Fig. 4). LPA (10 μM)-induced SRE activation was also blocked completely by C3 transferase. Again, this result indicates the important role of RhoA in the signaling pathway of ET-1 to SRE in Rat-2 cells. In summary, we report here that RhoA activity is critical in the ET-1-induced signaling cascade to *c-fos* SRE activation in Rat-2 fibroblast cells.

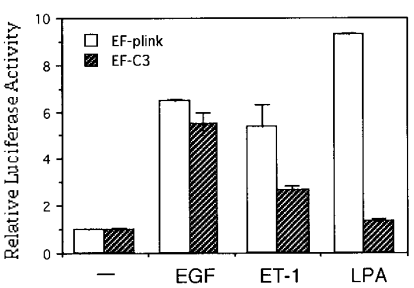

FIG. 4. Inhibition of ET-1-induced SRE activation by C3-transferase. A reporter gene plasmid, pSRE-WT-Luc (3 μg) was transiently co-transfected with pEFplink (0.75 μg) or pEFC3 (0.75 μg). Total amounts of DNA were kept at 20 μg with calf thymus carrier DNA. Transfected cells were serum-deprived in 0.5% FBS/DMEM for 36 hr before ET-1 (1 nM), EGF (50 ng/ml), or LPA (10 μM) treatment. After 2 hr of treatment, the relative activation of promoter activity to no treatment (—) was calculated as described in materials and methods and histograms of the results of the luciferase are shown.

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